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Research Article

Inhibition of DNA Methyltransferase Activates Tumor Necrosis Factor α–Induced Monocytic Differentiation in Acute Myeloid Leukemia Cells

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Abstract

Transcriptional silencing via promoter methylation of genes important for cell growth and differentiation plays a key role in myeloid leukemogenesis. We find that clinically achievable levels of 5-aza-2′-deoxycytidine (5-AZA-dC), a potent inhibitor of DNA methylation, can modify chromatin and restore the ability of tumor necrosis factor α (TNFα) to induce monocytic differentiation of the acute myeloid leukemia cells NB4 and U937. Although 5-AZA-dC cannot fully induce differentiation, we show that 5-AZA-dC acts directly on TNFα-responsive promoters to facilitate TNFα-induced transcriptional pathways leading to differentiation. 5-AZA-dC regulates the expression of *Dif-2*, a TNFα target gene, by deacetylating chromatin domains in a methylation-dependent manner. Chromatin immunoprecipitation analyses of the *Dif-2* promoter show histone hyperacetylation and a recruitment of the nuclear factor-κB transcription factor in response to 5-AZA-dC. Furthermore, 5-AZA-dC plus TNFα enhances the level of phosphorylated RNA Pol II at the *Dif-2* promoter via synergistic recruitment of TFIIH. We conclude that nonspecific changes in chromatin can allow a specific transcriptional inducer to overcome blocks in leukemic cell differentiation. Our results support the concept of low doses of 5-AZA-dC acting in combination with other agents to target epigenetic changes that drive malignant growth in leukemic cells. [Cancer Res 2009;69(1):55–64]

Introduction

Epigenetics is the study of heritable changes in gene expression that are not accompanied by changes in the DNA sequence. Cytosine methylation at CpG dinucleotides is the only known epigenetic modification of DNA itself and has a role in the control of gene expression (1). CpG methylation silences genes by sterically impeding binding of transcription factors that recognize sequences containing CG or by recruiting repressor proteins that bind specifically to methyl-CpGs. Moreover, DNA methylation has a direct influence on histone acetylation and higher-order chromatin structure, favoring the compacted chromatin production that is refractory to transcription (2). DNA hypermethylation in the promoter of tumor suppressor genes (TSG) may play a crucial role in carcinogenesis (3).

The deoxycytidine analogue 5-aza-2′-deoxycytidine (5-AZA-dC) is widely used as a DNA methylation inhibitor to activate the expression of epigenetically silenced genes. Once incorporated into the DNA, 5-AZA-dC covalently traps DNA methyltransferases (DNMT) in the form of a covalent-DNA adduct. Consequently, cellular DNMTs are depleted, and genomic DNA is demethylated during continued DNA replication, resulting in replication-dependent DNA hypomethylation (4). The covalent enzyme-DNA adduct can be cytotoxic at high levels, but *in vitro* efforts aimed at understanding the mechanism of demethylating agents suggest that demethylation of TSG can occur at substantially lower concentrations than those required for a cytotoxic effect (5). 5-AZA-dC can reprogram leukemic cells to differentiate toward a mature phenotype (6); however, subpopulations may resist the 5-AZA-dC treatment, thereby remaining as immature precursors. One approach to overcome this is to use specific differentiating agents in combination with 5-AZA-dC.

Tumor necrosis factor α (TNFα) promotes differentiation of human myeloid leukemic cells along a monocyte/macrophage pathway (7). TNFα acts through cognate receptors (TNFR1 and TNFR2) that relay downstream signals through a proapoptotic pathway, mediated via TNFR1 (8), and a prosurvival/differentiation pathway, mediated via TNFR2 (9). Activation of TNFR2 stimulates a translocation of the nuclear factor-κB (NF-κB) family of transcription factors (p65, p52, p50, c-Rel, and RelB) into the nucleus. In most unstimulated cells, the NF-κB complex is kept in an inactive state in the cytoplasm by interacting with members of the IκB family of proteins, which mask the nuclear localization signal of NF-κB. TNFα stimulation rapidly induces IκB kinase β (IKKβ), leading to IκBα phosphorylation and degradation. This in turn allows NF-κB transcription factors to translocate into the nucleus, bind their response elements as homodimers or heterodimers, and activate target genes. The role of NF-κB as a tumor promoter is well described, as its activation correlates with metastasis, angiogenesis, and a prosurvival/pro-proliferation phenotype (10). However, a growing body of evidence is emerging supporting NF-κB as a tumor suppressor (11), as it has been linked to p53-mediated apoptosis (12), a reduced angiogenic/metastatic potential in epithelial cells and mouse embryonic fibroblasts (13), and differentiation of leukemia cells as shown by our lab and other groups (14).

Acute myeloid leukemia (AML) is characterized by a block in terminal differentiation of hematopoietic precursors and repression

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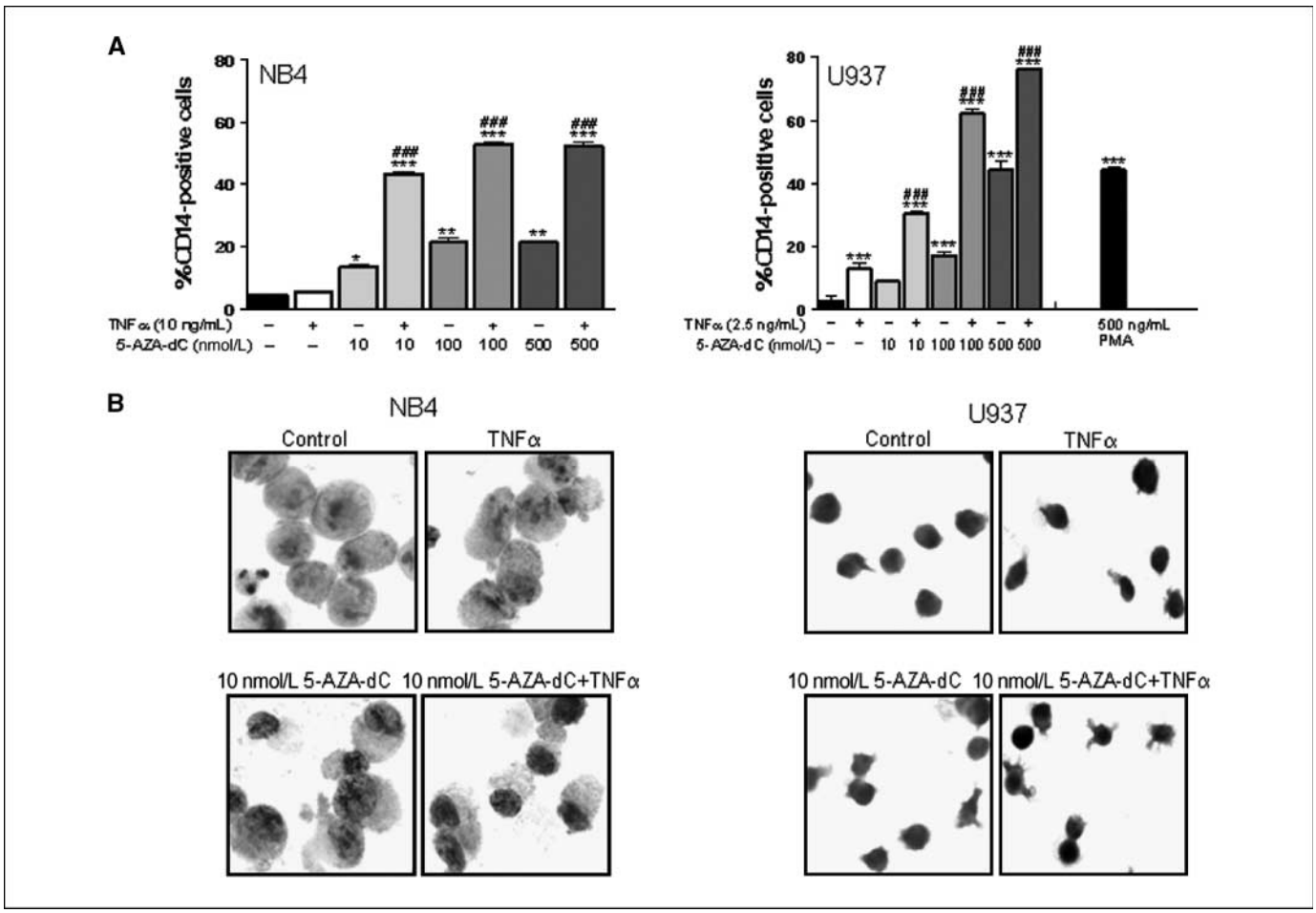

Figure 1. 5-AZA-dC enhances TNFα-induced monocytic differentiation in NB4 and U937. NB4 and U937 cells were treated for 48 h with 5-AZA-dC and TNFα. *A,* cytofluorometric analysis of cell surface marker expression; percentages of cells expressing monocyte-specific CD14 determined using monoclonal FITC-labeled antibodies. Results are representative of one of three experiments performed in triplicate. *Columns,* average of three independent samples; *bars,* SD. *Asterisks,* significant difference (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) from control. *Number signs,* significant difference (###, $P < 0.001$) from 5-AZA-dC–treated or TNFα-treated cells. *B,* morphologic analysis of representative NB4 cells treated with TNFα and 10 nmol/L 5-AZA-dC for 48 h. Cells were stained with May-Grunwald/Giemsa and viewed at ×650 magnification.

of normal hematopoiesis. AML is often caused by specific chromosomal translocations, such as t(15;17) in acute promyelocytic leukemia (APL), resulting in the generation of a chimeric oncogene acting as a transcriptional repressor (15, 16). The driving oncogene behind APL, PML/retinoic acid receptor (RAR) α, represses transcription of RAR target genes required for cellular differentiation via the recruitment of corepressive complexes that include histone deacetylases as well as DNMTs. Treatment of APL cells, as well as patients, with all-*trans* RA (ATRA) can overcome the transcriptional repression and induce granulocytic differentiation. In addition, our lab has previously shown that ATRA can act to overcome resistance to the differentiation stimulus of TNFα in APL and non-APL AML cells (14, 17). Surprisingly, we found that the combination of TNFα and ATRA did not induce granulocytic differentiation via RAR-dependent transcriptional pathways (18). Instead, ATRA enhanced the induction of TNFα target genes involved in monocytic differentiation through remodeling of chromatin to facilitate binding of other transcription factors, including NF-κB, to their response elements (19).

In this study, we asked whether another chromatin remodeling agent, 5-AZA-dC, could induce chromatin changes at the promoters of TNFα-responsive genes to potentiate or reactivate the promonocytic actions of TNFα in AML cells. This is significant as there are tumor types where ATRA is ineffective, whereas the broad activity of 5-AZA-dC may translate into a broader applicability. We show that low doses of 5-AZA-dC in combination with TNFα induce monocytic differentiation in the well-characterized APL cell line NB4 as well as in the non-APL, promonocytic cell line U937. We show that 5-AZA-dC cooperates with TNFα to activate transcription via DNA demethylation and histone modifications. This facilitates binding of the TNFα-activated transcription factors p65/p50 to previously inaccessible DNA-binding sites, leading to enhanced recruitment of phosphorylated RNA Pol II and, thus, gene expression. Importantly, we show for the first time that a very low dose, 10 nmol/L, of 5-AZA-dC is able to induce a time-dependent depletion of DNMT1 along with DNA demethylation and histone acetylation at the promoter of a TNFα target gene.

Materials and Methods

Materials. RPMI 1640 and fetal bovine serum (FBS) were purchased from Invitrogen. 5-AZA-dC, trichostatin A, and phorbol 12-myristate

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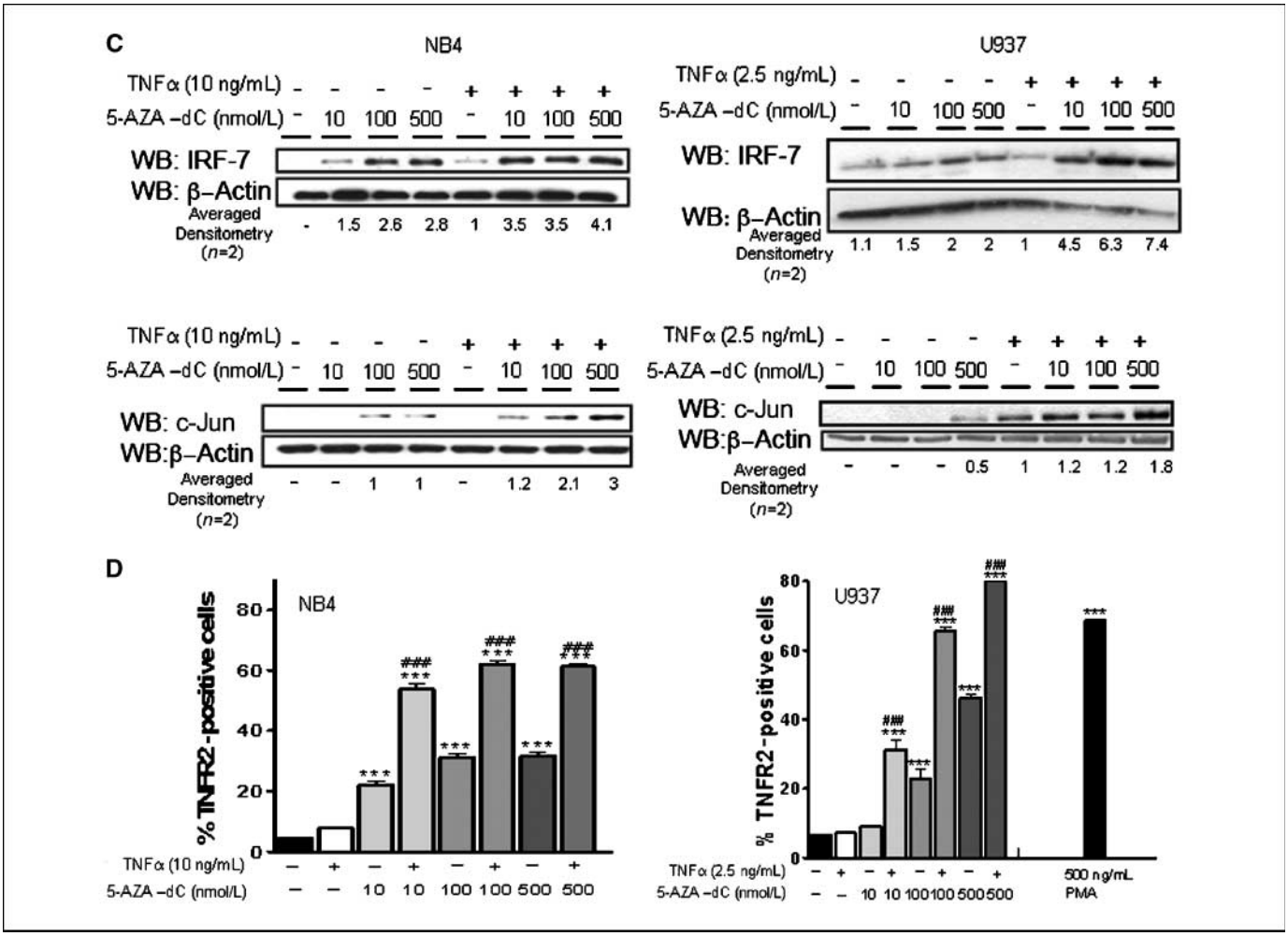

Figure 1. *Continued*. *C,* regulation of transcription factors involved in monocytic differentiation in NB4 and U937 cells. Western blotting was performed to determine IRF-7 and c-Jun protein levels with β-actin as loading control. Densitometry analyses of blots were performed using GeneTools software. Densitometry values were normalized to β-actin. The lowest normalized value then served as a reference for the ratio value presented. *D,* cytofluorometric analysis of TNFR2 levels on NB4 and U937 cells in response to 48-h treatment with 5-AZA-dC and TNFα; percentages represent the number of FITC-positive cells. *Columns,* average of three independent samples; *bars,* SD. *Asterisks,* significant difference (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) from control. *Number signs,* significant difference (###, $P < 0.001$) from 5-AZA-dC–treated or TNFα-treated cells.

13-acetate (PMA) were obtained from Sigma, dissolved in PBS, and stored at −80°C. Recombinant TNFα (PeproTech) was dissolved in sterile PBS. [γ-^{32}P]ATP was purchased from Perkin-Elmer.

Cell culture. NB4 and U937 were maintained in RPMI 1640 supplemented with 10% FBS. Cells were treated with 2.5 or 10 ng/mL of TNFα and 10, 100, and 500 nmol/L of 5-AZA-dC for 48 h, unless otherwise specified. For all experiments, control measurements were obtained from vehicle-treated cells.

Differentiation assay. Nitroblue tetrazolium (NBT) reduction assay and fluorescence-activated cell sorting (FACS) analysis for differentiation markers CD14 and CD11b were performed as previously described (17). Morphology was evaluated using May-Grunwald/Giemsa–stained cytosmears.

TNFR1 and TNFR2 expression. TNFR1 and TNFR2 were detected by FACS using FITC-labeled antibodies (R&D Systems). Fluorescence was measured on a Becton Dickinson FACS.

Western analysis. Fifty micrograms of nuclear extracts, prepared as previously described (17), were used for Western blotting to detect DNMT1 (MethylGene, Inc.), IRF-7, c-Jun (Santa Cruz Biotechnology), and total acetylated H3, H4, H3 dimethyl arginine 17, acetylated H3 lysine 14-9, and H3 trimethyl lysine 4 (Upstate Biotechnology). β-Actin (Sigma) confirmed equal protein loading.

mRNA analysis. Total RNA was isolated using Trizol as previously described (20). For Northern blotting, 20 μg RNA was electrophoresed on a 1% formaldehyde agarose gel and blotted onto Zeta probe (Bio-Rad) transfer membranes. cDNA probes were labeled by random priming (Pharmacia-Amersham). Hybridization and autoradiography were performed as previously described (18). *Dif-2* full-length cDNA was a kind gift from Dr. John Hiscott (Lady Davis Institute for Medical Research, Montreal, Canada).

Quantitative real-time PCR. cDNA was prepared from 5 μg total RNA using SuperScript II reverse transcriptase (Invitrogen). The relative quantity of *Dif-2* and DNMT1 mRNA was measured using the Applied Biosystems 7500 Fast Real-time PCR System, with glyceraldehyde-3-phosphate dehydrogenase as the endogenous control. Amplification was performed with 40 cycles of 95°C for 15 s and 60°C for 60 s using the following primers: 5′-CGCTCTGGACCTCAGCACTT-3′ (forward) and 5′-TGTTTCTTTGTGGTTTTTCGGATT-3′ (reverse) for *Dif-2* and 5′-AGGGAAAAGGGAAGGGCAAG-3′ (forward) and 5′-AGAAAACACATCCAGGGTCCG-3′ (reverse) for DNMT1. A SYBR Green–based detection method was used (Applied Biosystems).

Luciferase assay. Cells (5×10^6) were electroporated (350 V; 950 μF) with 5 μg Igκ κB-containing pBIIX luciferase report construct (21). Assays were performed as recommended by the manufacturer (Promega) using a

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Lumat LB 9507 luminometer (EG&G Berthold). Values were normalized with respect to protein content of the cell lysates to obtain the relative luciferase activity. Each transfection was performed in triplicate and repeated thrice.

Chromatin immunoprecipitation. The Upstate Biotechnology chromatin immunoprecipitation (ChIP) protocol was used with minor modifications, as summarized in Supplementary Materials and Methods.

Quantitative high-throughput analysis of DNA methylation by base-specific cleavage and mass spectrometry. Genomic DNA (500 ng) was treated with sodium bisulfite using the EZ DNA Methylation kit (Zymo Research) as per the manufacturer's protocol. Bisulfite-converted DNA was amplified using specific primers and submitted to base-specific cleavage and mass spectrometry using Sequenom MassARRAY EpiTyper as described in Supplementary Materials and Methods.

Statistical analysis. Significance was determined by ANOVA followed by Newman-Keuls post-tests using Prism version 4.0 (GraphPad).

Results

5-AZA-dC and TNFα induce monocytic differentiation in NB4 and U937 cells. Previously, we showed that addition of ATRA to TNFα modifies chromatin to overcome resistance to TNFα-mediated monocytic differentiation in AML cell lines (20, 21). Thus, we asked whether 5-AZA-dC could cooperate with TNFα to induce differentiation in TNFα-resistant AML cell lines. NB4 and U937 cells were treated for 48 hours with low doses of 5-AZA-dC (10, 100, and 500 nmol/L) and doses of TNFα that had little or no effect (10 ng/mL in NB4 and 2.5 ng/mL in U937), separately or in combination. FACS analysis of NB4 cells showed that the expression of CD14, a marker of monocytic differentiation, did not significantly increase after TNFα treatment alone, whereas 5-AZA-dC caused a modest increase (Fig. 1*A, left*). The combination of 5-AZA-dC with TNFα was a more effective inducer of differentiation than either agent alone, substantially increasing the percentage of CD14-positive cells. Similarly, U937 cells treated with 5-AZA-dC exhibited a dose-dependent increase in the percentage of CD14-positive cells that was enhanced with the addition of TNFα (Fig. 1*A, right*). Of note, U937 cells were more sensitive than NB4 to TNFα alone, and even at a lower concentration, a weak increase in CD14 was observed. Interestingly, TNFα plus 500 nmol/L 5-AZA-dC led to an increase in the percentage of CD14-positive cells to >80%, which is significantly greater than that observed with PMA, a well-known powerful inducer of monocytic differentiation in U937 (22, 23).

There was no induction of CD11b, a granulocytic differentiation marker, by either TNFα or 5-AZA-dC, alone or in combination (data not shown). To confirm that the cells differentiate into a monocytic phenotype, we evaluated morphologic changes of NB4 and U937 cells treated with TNFα and 5-AZA-dC for 48 hours (Fig. 1*B*). NB4 cells treated with 10 nmol/L 5-AZA-dC + TNFα display a lower nucleus to cytoplasmic ratio than control cells, chromatin condensation, and irregular cytoplasmic contours, typical of differentiated monocytes (24). TNFα alone had no discernible effect on cell morphology, whereas 5-AZA-dC–treated cells start to show a decreased nucleus to cytoplasm ratio and irregular contours. U937 cells treated with 10 nmol/L 5-AZA-dC + TNFα showed irregular contours with developed pseudopods, characteristic of a monocytic/macrophage phenotype (25), whereas TNFα or 5-AZA-dC alone did not induce morphologic changes. NBT analysis indicated that 5-AZA-dC and TNFα alone or in combination did not induce any significant ability to produce oxidative bursts (percent NBT^+ cells, <5%) in NB4 and U937 (data not shown), suggesting that the treatment-derived monocytes are not fully functional. Of note, no significant cell death was detected after 48 hours of treatment with 5-AZA-dC or TNFα in either cell line, as determined by propidium iodide staining (<10% cells with sub-G_0 DNA content; data not shown).

We further analyzed the expression of two regulators of the monocytic differentiation pathway: IRF-7 (Fig. 1*C, left*; refs. 26, 27) and c-Jun (Fig. 1*C, left*; ref. 28). NB4 and U937 cells treated for 48 hours with 5-AZA-dC showed a dose-dependent increase of IRF-7 protein expression, whereas a small increase was observed with TNFα alone. In the presence of both 5-AZA-dC and TNFα, a greater increase in IRF-7 protein expression was observed. In NB4 cells, 48 hours of TNFα alone had no effect on c-Jun expression, 100 to 500 nmol/L of 5-AZA-dC induced a modest expression, and 5-AZA-dC plus TNFα substantially enhanced c-Jun expression at all concentrations studied. In U937 cells, TNFα alone increased c-Jun protein levels (Fig. 1*C, right*), consistent with partial induction of differentiation by TNFα alone in these cells. 5-AZA-dC alone modestly induced c-Jun expression at 500 nmol/L and increased c-Jun expression induced by TNFα (Fig. 1*C, right*).

We also evaluated the expression of TNFR1 and TNFR2 in NB4 and U937 cells by FACS. As previously shown for the combination of ATRA and TNFα (17), 5-AZA-dC induces an increase in TNFR2 expression that is significantly augmented in the presence of TNFα (Fig. 1*D*), whereas no increase in TNFR1 is seen (data not shown).

5-AZA-dC and TNFα cooperate to induce transcription of TNFα target genes. To investigate whether 5-AZA-dC, alone or in combination with TNFα, regulates transcription through NF-κB response elements, as we had shown previously with RA + TNFα (23), cells were transfected with a luciferase construct containing an Igκ κB. In NB4 cells, neither TNFα nor 10 nmol/L 5-AZA-dC induced the transcriptional activity of this reporter, but transcription was enhanced 4-fold by the cotreatment (Fig. 2*A*). In U937 cells, TNFα alone induced transcription, but this could be further increased by the addition of 5-AZA-dC (Fig. 2*B*). To confirm the relevance of these results, we studied the effect of 5-AZA-dC on the transcription of an endogenous gene containing NF-κB response elements. Induction of *Dif-2* (29), a TNFα target gene involved in monocytic differentiation that is synergistically activated by ATRA plus TNFα (17), was thus assessed after treatment with 5-AZA-dC and TNFα (Fig. 2*C* and *D*). NB4 and U937 cells were pretreated with 5-AZA-dC for 48 hours to allow for adequate 5-AZA-dC incorporation into DNA followed by TNFα for 2 hours. In NB4, TNFα alone for 2 hours did not induce *Dif-2* expression, consistent with a lack of differentiation response in these cells. 5-AZA-dC alone induced a dose-dependent increase in *Dif-2* mRNA, whereas the addition of 5-AZA-dC plus TNFα induced this gene more strongly (Fig. 2*C*). In U937, TNFα alone increased *Dif-2* mRNA, consistent with responsiveness of this cell line to TNFα, but the addition of 5-AZA-dC potentiated the response (Fig. 2*D*). Taken together, our results indicate that 5-AZA-dC potentiates activation of TNFα-responsive gene expression.

5-AZA-dC treatment alters translocation of p65 and facilitates recruitment of NF-κB to target promoters. TNFα-induced transcription is IKKβ dependent and can be inhibited by selective inhibitors of this kinase, such as compound A (30). To test whether the effect of 5-AZA-dC on *Dif-2* transcription is dependent on NF-κB/IKKβ, we assessed induction of *Dif-2* expression after 48-hour 5-AZA-dC treatment of NB4 in the presence or absence of compound A. Indeed, the inhibitor significantly reduced the induced levels seen with 5-AZA-dC (Fig. 3*A*). The inhibitor also reduced *Dif-2* induction by 5-AZA-dC plus TNFα (data not shown).

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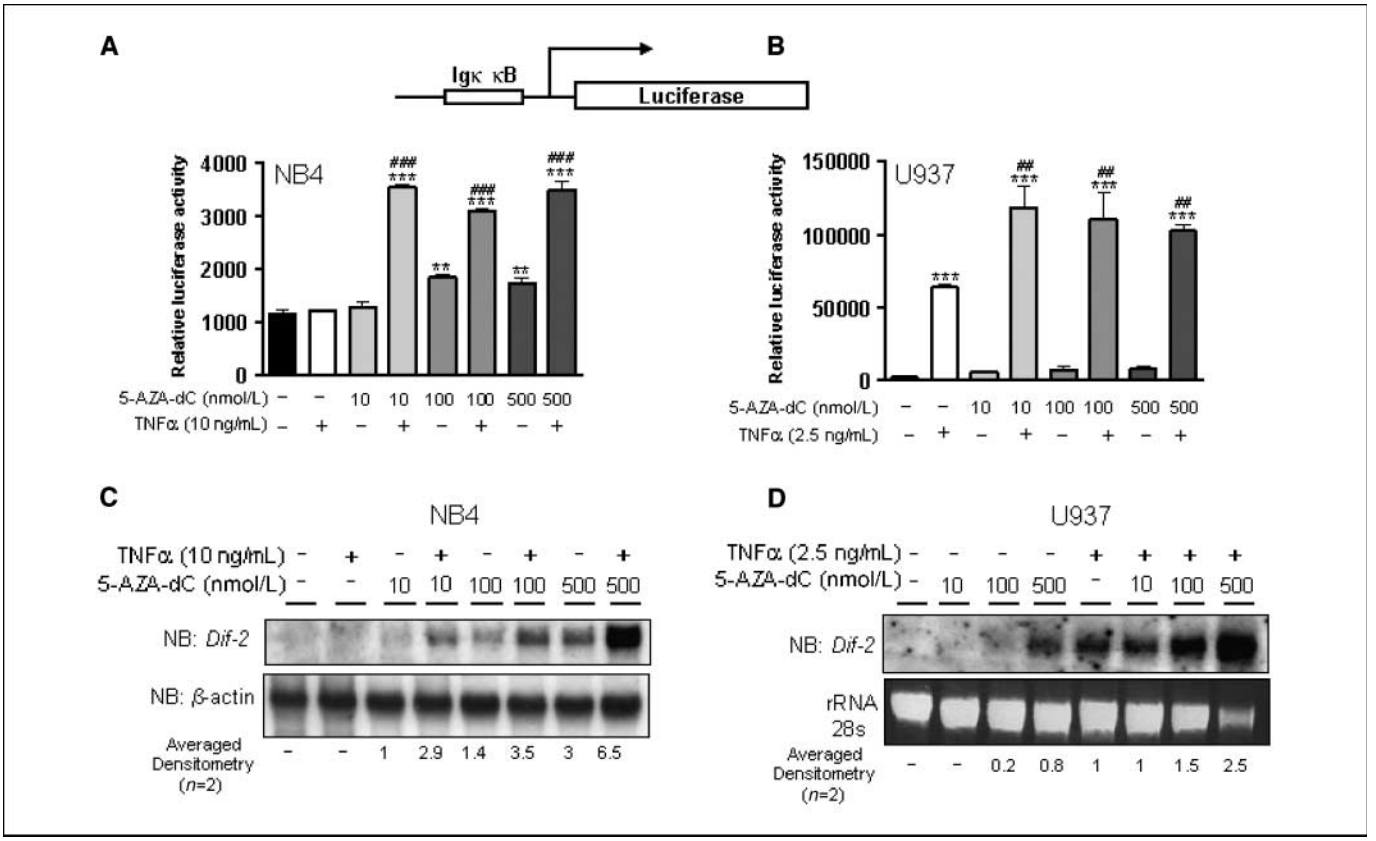

Figure 2. 5-AZA-dC promotes transcription through NF-κB response elements. *A* and *B,* NB4 and U937 cells were transfected by electroporation with Igκ κB constructs and treated for 48 h. Induction of luciferase activity by TNFα and 5-AZA-dC was quantified. After reporter gene activity had been measured, the values were normalized with respect to protein content of the cell lysates to obtain the relative luciferase activity. Each transfection was performed in triplicate and repeated thrice. *Columns,* average of three independent samples; *bars,* SD. *C* and *D, Dif-2* mRNA levels were examined by Northern blot. NB4 and U937 cells were treated for 48 h with 5-AZA-dC followed by 2-h treatment with TNFα. Total RNA (10 μg) was loaded in each lane and probed with *Dif-2* full-length cDNA. β-Actin and rRNA levels indicate equal loading. Densitometry analyses were performed on representative blots.

We next examined the effects of 5-AZA-dC on NF-κB signaling. Western blot analyses on fractionated NB4 cells for p65 showed a dose-dependent translocation of p65 from the cytoplasm to the nucleus after treatment with 5-AZA-dC (Fig. 3*B*). To assess whether this increase of p65 in the nucleus would translate into increased NF-κB binding to the *Dif-2* promoter (Fig. 3*C*), ChIP experiments were performed in NB4. These showed a significant increase in p65 promoter binding in cells treated for 48 hours with 10 nmol/L 5-AZA-dC (Fig. 3*D, left*) but, in agreement with previous data from our lab (19), not in cells treated with TNFα alone. When 5-AZA-dC and TNFα were combined, a significant increase in p65 binding was observed. Of note, both 5-AZA-dC and TNFα alone were able to recruit p50 to the *Dif-2* promoter (Fig. 3*D, right*).

5-AZA-dC depletes DNMT1 levels leading to DNA demethylation. The mechanism by which 5-AZA-dC induces demethylation is believed to require its incorporation into DNA followed by the formation of covalent complexes with DNMT(s), thereby depleting DNMT cellular pools (31). Therefore, we examined levels of DNMT1 in NB4 after treatment with 5-AZA-dC (Fig. 4*A*). Western blot analyses showed that the protein levels of DNMT1 started to decline after 24 hours of treatment with all tested concentrations of 5-AZA-dC and were undetectable after a 500 nmol/L treatment for 24 hours or a 100 nmol/L treatment for 48 hours. DNMT1 requires hemimethylated DNA as a substrate; therefore, the effect of 5-AZA-dC will not be fully exerted until the analogue-incorporated DNA undergoes replication. We confirmed that 2 hours of 5-AZA-dC treatment does not alter DNMT1 levels (Fig. 4*A*). To determine whether the decrease in DNMT1 levels was due to reduced gene expression, we analyzed mRNA levels of DNMT1 by real-time PCR. A small but significant increase in the mRNA levels of DNMT1 was observed in NB4 after 5-AZA-dC treatment (Fig. 4*B*), showing that the decrease in DNMT1 protein on 5-AZA-dC treatment was not due to transcriptional regulation.

The above results show that soluble DNMT1 depletion is achievable at the lowest dose of 10 nmol/L 5-AZA-dC, and imply that DNMT1 depletion is critical for demethylation and gene induction. To assess whether these effects were associated with DNA demethylation, a MassARRAY analysis was performed on the *Dif-2* gene in NB4 cells. MassARRAY provides a quantitative measurement of multiple CpG methylation sites over long sequences. The two different regions examined included a 362-bp region in the promoter and a 397-bp region within the *Dif-2* coding sequence (Fig. 4*C*). Among the CpGs detected by MassARRAY, a series of four consecutive sites all localized in the proximal promoter region were hypermethylated in untreated cells (methylation level = 82–100%). These CpGs are localized in proximity to potentially important gene regulatory elements (CACCC and CAAT boxes). In contrast, CpGs localized within the *Dif-2* coding sequence or more upstream were unmethylated (6–20% methylation).

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Exposure of NB4 cells to a 10 or 500 nmol/L dose of 5-AZA-dC reduced methylation in the regulatory region within 48 hours. Taken together, the above results suggest that the decrease in cytosine methylation at the *Dif-2* promoter region is due to titration of functional DNMT1 and that functionally significant DNMT1 depletion was attainable with only 10 nmol/L 5-AZA-dC.

5-AZA-dC–induced chromatin modifications allow increased Pol II accessibility to the *Dif-2* promoter region resulting in gene expression. Previous studies have shown that inhibition of DNA methylation leads to histone acetylation (32). To determine whether 5-AZA-dC induced general chromatin reorganization, we examined global levels of total acetylated H3 and H4, along with more specific modifications of H3 associated with an open and transcriptionally active chromatin. Western blot analysis revealed a dose-dependent increase in the levels of total acetylated H3 and H4 in NB4 cells treated with 5-AZA-dC for 48 hours (Supplementary Fig. S1). This effect requires incorporation into DNA, as a 2-hour treatment produced no increase in total acetylated H3 and H4 (Supplementary Fig. S2). Several specific modifications of H3 associated with a more transcriptionally active state also increased in response to 5-AZA-dC, in a dose-dependent manner, and this effect was enhanced by TNFα (Supplementary Fig. S3). We thus examined whether 5-AZA-dC modified histone acetylation at the *Dif-2* promoter. NB4 cells were examined by ChIP using polyclonal antibodies against acetylated H3 and H4, and 10 nmol/L 5-AZA-dC was sufficient to induce acetylation of both H3 and H4 (Fig. 5*A*). However, 10 nmol/L 5-AZA-dC can induce *Dif-2* expression only in the presence of TNFα (see Fig. 2*C*). Thus, we further examined the effects of 5-AZA-dC and TNFα alone and in combination on the *Dif-2* promoter. NB4 cells were used due to their reduced sensitivity to TNFα, thereby allowing for a greater appreciation of the synergy between 5-AZA-dC and TNFα. We first examined the possibility of an enhanced recruitment of the preinitiation complex protein Pol II to the *Dif-2* promoter in the presence of both drugs. ChIP analysis showed a recruitment of Pol II in response to either 5-AZA-dC or TNFα alone, although maximal promoter recruitment of Pol II was observed with the combination of TNFα and 100 nmol/L 5-AZA-dC (Fig. 5*B, left*). Phosphorylation of the COOH-terminal repeat of Pol II plays a crucial role in transcriptional elongation, and it has been reported that NF-κB binding (33) can stimulate Pol II phosphorylation. We investigated the status of Pol II phosphorylation at the *Dif-2* promoter and found no significant induction of Ser^{205} phosphorylation of Pol II in cells treated with 5-AZA-dC or TNFα alone, whereas the combination caused a significant increase (Fig. 5*B, right*). Similarly, only the combination recruited the p62 subunit of the Pol II kinase, TFIIH (Fig. 5*C*), which can mediate Pol II phosphorylation (34). We then examined acetylation of histone H3 lysine 14, which has been linked to transcriptional activation, at the *Dif-2* promoter. Again, consistent with the above results, the combination 5-AZA-dC plus TNFα resulted in a strong synergistic increase of acetyl H3 lysine 14, whereas no increase was observed with either drug alone (Fig. 5*D*). Our results show not only that DNA methylation is required to maintain silencing of *Dif-2* but also that the erasure of the DNA methylation signature leads to specific histone modifications that facilitate binding of TNFα-induced

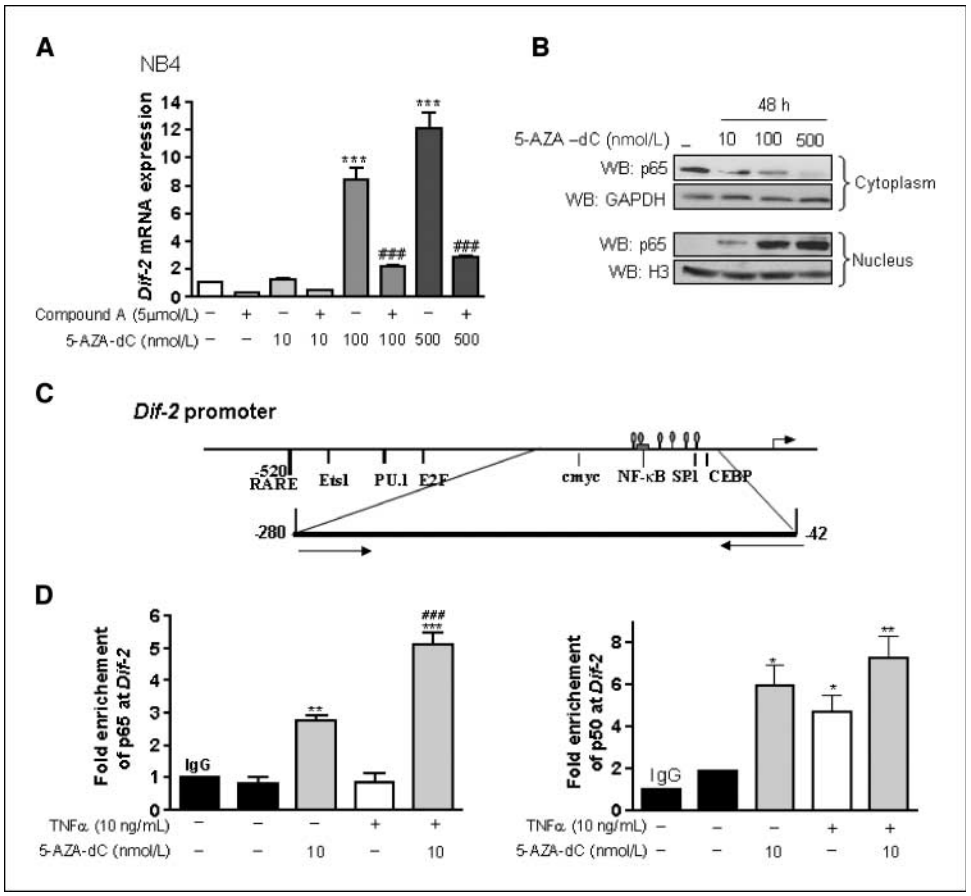

Figure 3. Activation of *Dif-2* expression is NF-κB dependent. *A,* quantitative real-time PCR analysis of *Dif-2* expression in NB4 treated for 48 h with 5-AZA-dC in the presence or absence of an IKKβ inhibitor (compound A, 5 μmol/L). *Columns,* average of three independent samples; *bars,* SD. *Asterisk,* significant difference (***, $P < 0.001$) from control. *Number signs,* significant difference (###, $P < 0.001$) from 5-AZA-dC–treated cells. *B,* p65 transcription factor levels were measured by Western blot; cytoplasmic and nuclear cell extracts were prepared from NB4 cells treated or not with 5-AZA-dC. *GAPDH,* glyceraldehyde-3-phosphate dehydrogenase. *C,* overview of the *Dif-2* promoter region showing putative transcription factor binding sites. *Arrows,* position of primers used in ChIP experiments. The transcription start site (TSS) was set as +1. *D,* ChIP analyses of the *Dif-2* promoter region of NB4 cells cultured with or without 10 nmol/L 5-AZA-dC for 48 h followed by 1-h treatment with TNFα. Chromatin was immunoprecipitated with antibodies specific for p65 and p50 NF-κB transcription factors and rabbit or mouse IgGs as isotype antibody control. The immunoprecipitated chromatin was amplified with real-time PCR. *Columns,* mean of six measurements (see Materials and Methods); *bars,* SD. *Asterisks,* significant difference (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) from control. *Number signs,* significant difference (###, $P < 0.001$) from 5-AZA-dC–treated or TNFα-treated cells.

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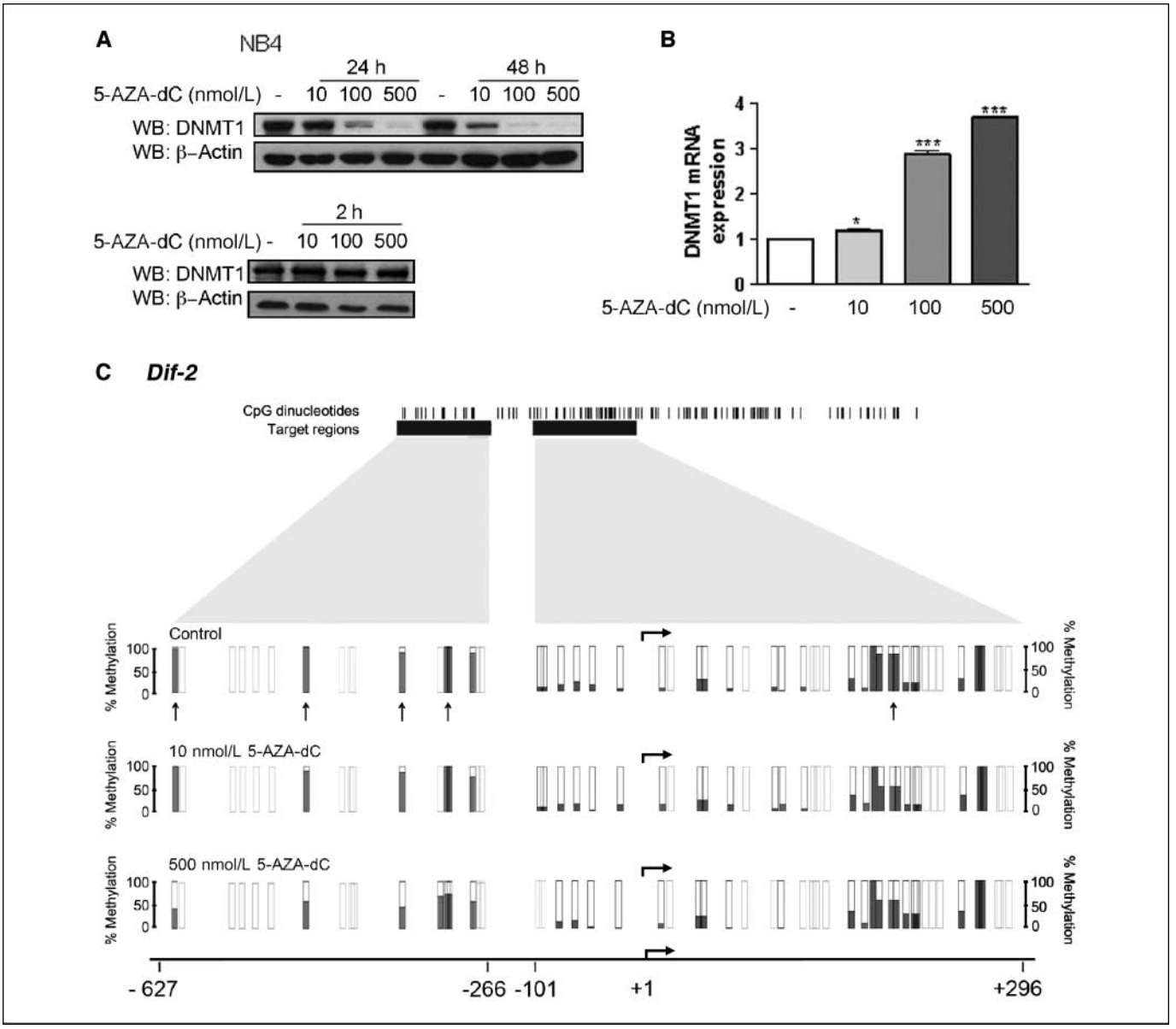

Figure 4. Effects of 5-AZA-dC on DNMT1 levels and DNA methylation. *A,* Western blot showing levels of DNMT1 protein after 5-AZA-dC treatment in NB4 cells. 5-AZA-dC treatment for 2 h is not able to deplete DNMT1 levels. β-Actin was used as loading control. *B,* DNMT1 mRNA expression from NB4 cells treated or not with 5-AZA-dC for 48 h was measured by quantitative real-time PCR. *Columns,* average of three independent samples; *bars,* SD. *C,* quantification of *Dif-2* gene methylation by MassARRAY analysis. Genomic DNA from untreated NB4 cells and NB4 cells treated for 48 h with 10 and 500 nmol/L of 5-AZA-dC was exposed to sodium bisulfite. Two regions in the vicinity of the TSS were selectively amplified and cytosine methylation levels were assessed using MassARRAY. The two regions analyzed encompassed 397 bp around the TSS (−101 to +296) and 362 bp upstream of the TSS (−627 to −266). The two MassARRAY target regions are depicted at the top of the figure. *White columns,* each cytosine analyzed (percent methylation for each cytosine is represented by the amount of filling of each column); *light gray columns,* CpGs not analyzed due to the limitations of the assay. Results are representative of one of two experiments performed in duplicate.

transcription factors and recruitment of the Pol II kinase TFIIH to form a fully active transcription complex.

Discussion

The cytosine analogue 5-AZA-dC is a potent inhibitor of DNMTs that is widely used *in vitro* as a demethylating agent and has undergone clinical trials (phases II and III) in the treatment of leukemia and hemoglobinopathy. Current clinical trials are investigating lower doses for longer durations to optimize DNA incorporation and to favor methylation reversal over cytotoxic effects. Recent interest in combining 5-AZA-dC with other epigenetic therapies and/or cytotoxic agents proposes 5-AZA-dC as a biological response modifier to increase the efficacy of other drugs. Here, we assessed whether 5-AZA-dC could increase the efficacy of TNFα. Therapies with recombinant TNFα (rTNFα) show antitumorigenic effects. However, severe toxicities are associated with rTNFα at therapeutically efficacious doses and have ultimately hindered its clinical development. Nonetheless, more targeted strategies are currently being pursued in the clinic to reduce TNFα-associated toxicities (35). Here, we use a strategy of combining TNFα with 5-AZA-dC to reduce the dose of TNFα necessary to illicit an effect.

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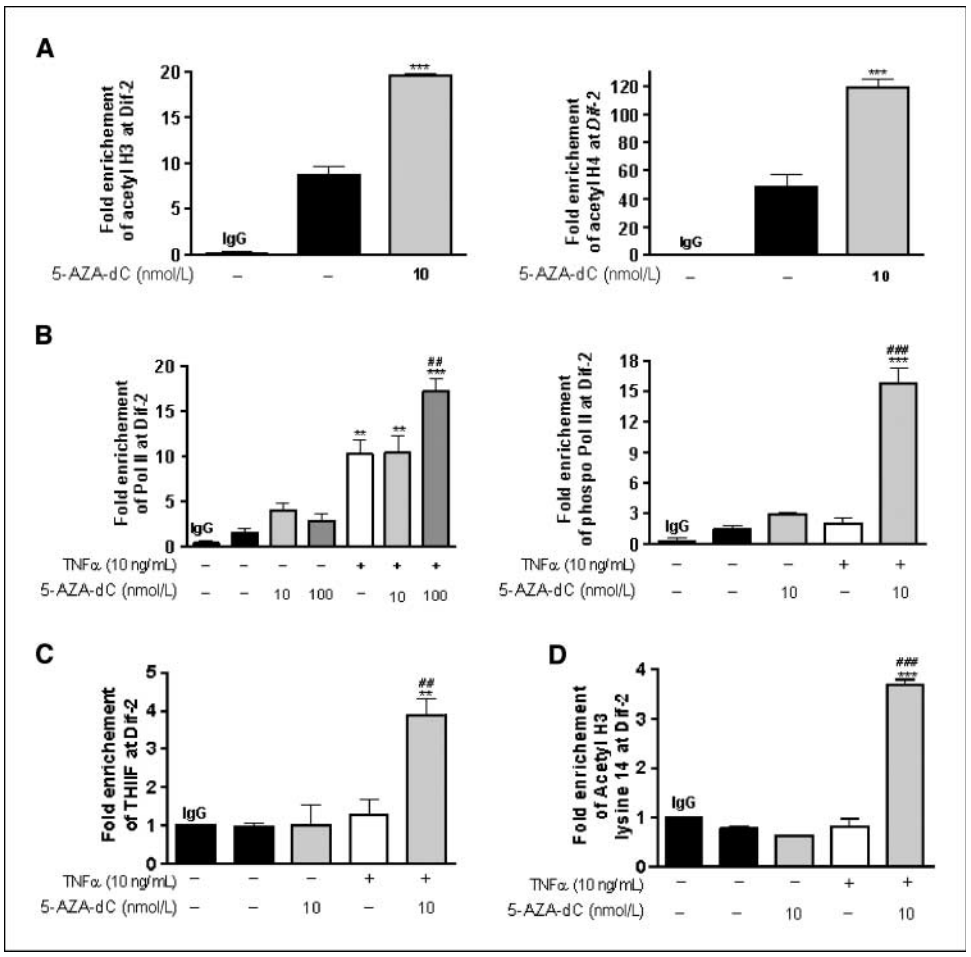

Figure 5. Epigenetic modifications induced by 5-AZA-dC at the *Dif-2* promoter region. ChIP analyses of *Dif-2* promoter region of NB4 cells cultured with or without 10 nmol/L 5-AZA-dC for 48 h and 1-h treatment with TNFα. Chromatin was immunoprecipitated with antibodies specific for acetylated H3 and acetylated H4 (*A*), Pol II and phospho-Pol II (*B*), TFIIH (*C*), and acetylated H3 lysine 14 (*D*). *Columns,* mean of six measurements (see Materials and Methods); *bars,* SD. *Asterisks,* significant difference (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) from control. *Number signs,* significant difference ($P < 0.001$) from 5-AZA-dC–treated or TNFα-treated cells. IgG indicates ChIP performed using rabbit or mouse IgGs as an isotype antibody control.

Consistent with our previous work that ATRA acts on chromatin of TNFα-responsive promoters to synergize with TNFα in APL cells (19), this study used low doses of 5-AZA-dC in the APL cell line NB4 and the monoblastic cell line U937 to potentiate the prodifferentiation effect of TNFα. This effect requires nuclear translocation of NF-κB, a transcription factor that can both promote and suppress malignant growth (11, 14). However, TNFα alone had little effect on the maturation of NB4 or U937 cells after 48 hours. We found that treatment with 5-AZA-dC followed by TNFα resulted in a much stronger induction of monocytic differentiation, as shown by increased CD14 expression along with cytologic changes and the up-regulation of c-Jun and IRF-7. This is consistent with our findings that 5-AZA-dC can specifically influence the activity of the TNFα pathway and supports the idea that 5-AZA-dC may potentiate other differentiating agents. Of note, our results show that costimulation with 5-AZA-dC and TNFα results in increased expression of TNFR2 without an accompanying increase in TNFR1 levels, consistent with the observation that TNFR2 is the major effector of the prodifferentiation/antiapoptotic NF-κB pathway, whereas TNFR1 may play a more proapoptotic role (36).

We have begun to elucidate a mechanism whereby 5-AZA-dC primes cells for TNFα-driven differentiation. We showed that 5-AZA-dC alone modified chromatin through DNA demethylation to induce the expression of genes involved in TNFα signaling. We found that 5-AZA-dC can synergize with TNFα to enhance the expression of an important TNFα target gene, *Dif-2* (29). *Dif-2* encodes an antiapoptotic protein that is specifically expressed in monocytes (37, 38), and may thus be directly involved in the differentiation process stimulated by 5-AZA-dC in combination with TNFα. 5-AZA-dC demethylated the *Dif-2* promoter and induced several histone modifications associated with active transcription specifically at the *Dif-2* promoter. Our data are consistent with other studies showing that DNA methylation targets histone modifications to gene promoters (39, 40) and that the maintenance of epigenetic silencing and of associated histone modifications is dependent on DNA methylation. It is important to emphasize that although the lowest tested dose of 5-AZA-dC (10 nmol/L) was able to induce some chromatin changes associated with a more open structure, thereby allowing NF-κB transcription factors to bind their response elements, it was only when 5-AZA-dC was combined with TNFα that we observed recruitment of the TFIIH complex, maximal Pol II phosphorylation, and thus gene transcription. Therefore, in addition to the reversal of repressive chromatin organization by 5-AZA-dC, a specific activating stimulus, here provided by TNFα, is required to fully induce transcription.

The accumulation of p65 in the nuclei of cells treated with 5-AZA-dC and the inhibition of 5-AZA-dC–induced *Dif-2* expression in the presence of an IKKβ inhibitor suggest that 5-AZA-dC exerts a direct, activating effect on the TNFα-signaling pathway. Although this may be an off-target effect of the drug, it may explain the ability of 5-AZA-dC to induce expression of a luciferase reporter

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gene under the control of an unmethylated NF-κB promoter construct (Fig. 2*A*) and does in part explain the recruitment of the NF-κB transcription subunits at the *Dif-2* promoter after 5-AZA-dC treatment. The exact mechanism of increased p65 nuclear translocation in response to 5-AZA-dC needs to be investigated, but we speculate that it may represent a 5-AZA-dC off-target effect, such as activation of kinases that mediate p65 nuclear shuttling.

A recent study from Robert and colleagues (41) indicated that DNMT1 is necessary and sufficient to maintain global methylation and aberrant CpG island methylation in human cancer cells. The use of either antisense or small interfering RNA to deplete DNMT1, but not DNMT3A or DNMT3B, potentiated the ability of 5-AZA-dC to reactivate silenced tumor-suppressor genes, indicating that inhibition of DNMT1 is the principal means by which 5-AZA-dC reactivates genes. In accordance, our data show that 5-AZA-dC induces a dose- and time-dependent depletion of DNMT1. Of note, we found that 2 hours of 5-AZA-dC treatment does not alter DNMT1 levels, suggesting that the effect of 5-AZA-dC will not be fully exerted until the DNA undergoes replication and incorporation of the analogue. We suggest that DNMT1 depletion is due, as previously proposed, to the trapping of the enzyme by the DNA-incorporated 5-AZA-dC (42). However, a role of microRNAs in regulation of DNMTs and methyl-binding protein at a posttranscriptional level has also been proposed (43, 44).

Importantly, the concentrations of 5-AZA-dC used here are in the range of plasma levels of this analogue used in clinical trials (45). Schrump and colleagues (46) showed that steady-state plasma concentrations of approximately 25 to 40 nmol/L were achieved during prolonged 5-AZA-dC infusion in patients with thoracic cancers. Their published preclinical studies indicated that a 5-AZA-dC concentration of 50 nmol/L was sufficient for NY-ESO-1 induction in cultured lung cancer cells (47). The concentrations used for TNFα (2.5 and 10 ng/mL) are also well within achievable serum concentrations as reported by Feinberg and colleagues (48). We now show that 10 nmol/L 5-AZA-dC is adequate to cause demethylation at specific gene promoters and, in the presence of TNFα, induce epigenetic reprogramming and differentiation. The sensitizing of leukemia cells to TNFα is significant as it takes a strategy used by Witcher and colleagues (49), and builds on it by replacing ATRA as a chromatin modulator with 5-AZA-dC, a drug with a broader applicability. That is, 5-AZA-dC could be used in malignancies resistant to ATRA. For example, 5-AZA-dC has shown encouraging results in solid tumor models, including breast cancer cell lines, whereas the potential of ATRA may be restricted to certain subtypes of leukemia. Our findings show that low doses of 5-AZA-dC are sufficient to induce chromatin reorganization of a hypermethylated and thus silenced gene into a more transcriptionally permissive state. Furthermore, we show that these changes can sensitize chromatin to additional modifications induced by specific differentiating agents, such as TNFα. We thus provide a mechanism for the synergistic induction of gene transcription and differentiation seen in AML cells treated with 5-AZA-dC and TNFα. The fact that 5-AZA-dC demethylates DNA in a global manner, and also induces global changes to chromatin, implies that this strategy could be applied to a wide variety of differentiating agents and their respective gene targets. The DNA demethylation observed in this study with a low and thus clinically relevant dose of 5-AZA-dC along with its ability to potentiate the prodifferentiation effect of TNFα provides support for the further development of combination therapies with low doses of 5-AZA-dC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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